Studies on the Transmissibility and Cytology of the Renal Carcinoma of *Rana pipiens* *

MARIA E. ROBERTS

(Department of Pathobiology, School of Hygiene and Public Health, The Johns Hopkins University, Baltimore, Maryland)

SUMMARY

The incidence of renal carcinoma in frogs maintained in the laboratory was not significantly affected by the inoculation of cell suspensions or cell-free filtrates of tumors. There was no significant difference in the tumor incidence in experimental or control frogs whether maintained individually or in groups of ten.

Histological sections of 61 tumors stained with Feulgen and fast green revealed that there were two types, designated here as Types I and II. Type I tumors consisted of enlarged cells, the nuclei of which contained a large amount of intensely staining Feulgen-positive material. Mitotic activity was seen in every tumor of this type, and a spherical or oval, dense, Feulgen-positive body was observed in the cytoplasm of many of the cells.

The cells of Type II tumors were also enlarged but appeared to be necrotic or degenerating. The nuclei stained faintly with Feulgen, and the chromatin material was very sparse. Mitotic activity was rarely seen in these tumors. In every tumor of this type some cells exhibited an irregularly shaped nuclear aggregate which stained intensely with fast green.

Of the 61 tumors examined histologically, 54 were Type I, and seven were Type II. All the Type I tumors were obtained from frogs that had been kept at 20°-25° C. since their arrival in the laboratory. All the Type II tumors were obtained from frogs which had been kept at 5° C. without being fed for at least 2 months (a state resembling hibernation). The acidophilic nuclear aggregate which appears in Type II tumors was not observed in Type I tumors.

The data which have been considered as evidence that a virus is involved in the etiology of the renal adenocarcinoma of *Rana pipiens* stem from two main lines of investigation: studies of transmissibility and of histology. Lucké (4, 5)

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found that 21.3 per cent of frogs given inoculations of desiccated or glycerinated preparations of tumor tissue developed the tumor after 6 months of laboratory maintenance, whereas 6.8 per cent of un inoculated controls had the neoplasm. Duryee (1) observed a 17 per cent tumor incidence among frogs given inoculations of filtrates of a series of tumors, but the spontaneous occurrence of the tumor in the frogs used by him was not indicated. Rafferty and Rafferty (11) found that tumors ap-
Histological examination of this neoplasm has revealed alterations analogous to changes of known viral etiology. Lucké (4–6) described intranuclear acidophilic "inclusion bodies" in some cells of some tumors. Fawcett (2), investigating tumors containing such inclusions with the electron microscope, described virus-like particles in the cytoplasm and the nucleus of cells which contained nuclear inclusions. These particles were not seen in cells which had no intranuclear alteration or in cells of tumors in which the acidophilic nuclear aggregate did not appear. Tweedell (12) found no nuclear inclusions in tumor cells stained with acridine orange. He did, however, describe cytoplasmic inclusions which fluoresced as did the nuclear chromatin and were removable by prior digestion with deoxyribonuclease.

The present investigation was undertaken in an effort (a) to obtain more completely controlled data on the transmissibility of this tumor by inoculation and (b) to attempt to resolve the conflicting reports concerning the cytological appearance of this tissue.

MATERIALS AND METHODS

Transmission experiments.—Mature Rana pipiens were obtained from frog collectors in the Lake Champlain area of Vermont. After arrival in the laboratory the frogs were kept either at 5°C. in half-gallon bowls, or at 20°–25°C. in ten-gallon tanks or in liter jars. Penicillin and sulfadiazine were used to prevent spread of bacterial infections. Gloves were rinsed before each frog was handled. Frogs were placed in groups of ten in tanks of approximately ten frogs each. Water was changed daily, this being accomplished in such a manner that there was no contact with water or frogs from any other tank or jar. Frogs at 20°–25°C. were fed larvae of Tenebrio molitor twice weekly.

Frogs kept at 20°–25°C. under the above conditions were used in the transmission studies within 1 week after arrival in the laboratory. The inoculum was prepared by homogenization (by hand, with a Ten-Broek homogenizer) of a washed and minced portion of kidney tissue in sterile amphibian Ringer's saline (NaCl, 113 mM; KCl, 2.0 mM; CaCl2, 1.4 mM; glucose, 1.1 mM). One-half of the resulting cell suspension was centrifuged at 800 × g for 10 min. at 5°C., and the supernatant was filtered through a sterile Seitz bacteriological filter. Thus, a cell suspension and a cell-free filtrate were obtained from the same homogenate of normal or tumorous kidney for the inoculation of control or experimental animals, respectively. Frogs which received inoculations were given 0.5-ml. intraperitoneal injections intraperitoneally. At the time of inoculation they were handled with rubber gloves which were rinsed with 10 per cent formalin, then water. Gloves were rinsed before each frog was handled to avoid contamination among the individual experimental animals in this manner. At the termination of each experiment frogs were killed by decapitation, and the kidneys and other organs were examined macroscopically for tumors. All tumors and other areas thought worthy of further scrutiny were fixed in Zenker's fluid for histological examination. Differences among experimental groups were tested for significance by the \( x^2 \) method.

In the first transmission experiment 150 frogs were given inoculations and placed in tanks. Only 124 of these frogs survived a severe epidemic of "redleg" (attributed to Bacillus hydrophillus fuscus (8) shortly after the beginning of their laboratory maintenance. One-third of the frogs received an inoculum of normal kidney tissue; one-third were given injections of material from a tumor containing no acidophilic intranuclear inclusions (see below); and one-third with material from a tumor having some cells with such aggregates. One-half of the frogs in each of these groups received a cell-free preparation of the inoculum and the other half a cell suspension. Equal numbers of frogs were allocated to each experimental group, and the mortality from redleg did not differ significantly from group to group. Frogs surviving the bacterial infection were maintained for a period of 4 months.

In the second transmission experiment 240 frogs were included, but only 213 of these survived a similar bacterial infection. As in the previous experiment, equal numbers of frogs were allocated to each experimental group, and the mortality from redleg did not differ significantly from group to group. One-third of the frogs were not given inoculations. One-third of the frogs received an inoculation of material from normal kidney tissue, half of them a cell suspension and the other half a cell-free filtrate. The remaining frogs were given inoculations of material from a tumor exhibiting no nuclear inclusions. Of this group, half received a suspension of cells and the other half a cell-free filtrate. One-half of the frogs in each of these groups were kept in tanks of approximately ten frogs each, and the other half individually in jars. In this experiment, as in the previous one, the frogs were maintained for 4 months.

Cytological studies.—Material to be examined histologically was fixed in Zenker's fluid and processed for light microscopy. Paraffin sections 5μ thick were hydrolyzed in 1.0 N HCl at 60°C. for 8
minutes and placed in Schiff's leuchofuchsins reagent (Feulgen stain) for 2 hours. Thereafter, the sections were placed in three changes of 0.5 per cent K$_2$SO$_4$ for 10 minutes each. They were counterstained with 0.2 per cent fast green in 95 per cent alcohol and mounted. Small tumors and several representative portions of each large tumor were sectioned serially and all sections examined thoroughly after staining.

RESULTS

Transmission experiments.—The first transmission experiment was performed to obtain data comparing the tumor incidence in frogs given inoculations of material from tumors and those given material from normal kidneys. Further, it was intended to compare tumor incidence after inoculation of a cell suspension and a cell-free filtrate from each source.

The second transmission experiment was performed to increase the data comparing tumor incidence in frogs given inoculations of normal and tumorous material. In addition, this experiment included a group of frogs which received no inoculation for the following reason: if the etiological agent were a virus endemic in the population, it could be latent in morphologically normal tissue. Thus, uninoculated frogs were also studied because "normal" inoculum might contain the agent. Moreover, this experiment was designed to show whether there was any difference in the tumor incidence in frogs kept in groups and those kept individually. The results of the two experiments after 4 months of laboratory maintenance of the frogs are shown in Table 1. All tumors found at autopsy were histologically confirmed. There were no statistically significant differences in the tumor incidence among the various groups given or not given inoculations, grouped or isolated. It should be added that there was no difference observed in the susceptibility of male and female frogs to this tumor.

Cytological studies.—The tubular epithelium (proximal and distal) of the normal frog kidney is composed of cuboidal cells arranged in a single layer, as previously described by Lucké (4) and Fawcett (2). The cytoplasm of these cells stains uniformly with fast green or with eosin. The Feulgen-positive material is all in the nucleus, where it is present in numerous chromocenters. The chromocenters are usually spherical in shape, but one which is apparently ring-shaped is also seen in many nuclei. This is presumed to be the nucleolar organizer. Closely associated with this structure is a spherical or oval body which stains with fast green, presumably the nucleolus. In some cells more than one nucleolus appears to be present. Mitosis was not observed in sections of the fifteen pairs of normal kidney examined.

In total, 61 tumors were examined cytologically and were found to be as described by Lucké (4, 6) in the following respects: (a) they were composed

### TABLE 1

<table>
<thead>
<tr>
<th>EXPERIMENT</th>
<th>INOCULUM</th>
<th>None</th>
<th>Normal kidney</th>
<th>Tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. frogs alive</td>
<td>No. tumor-bearing frogs</td>
<td>Per cent of frogs with tumors</td>
<td>No. frogs alive</td>
</tr>
<tr>
<td>A. Grouped:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Cell-free</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. Isolated:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Cell-free</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. Combined:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Cells (A.1.+B.1.)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Cell-free (A.2.+B.2.)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D. Totals by inoculum (C.1.+C.2.)</td>
<td>72</td>
<td>15</td>
<td>21</td>
<td></td>
</tr>
</tbody>
</table>

Determinations were made at 4 months.
cells were very large compared with those of the normal kidney epithelium.

Further cytological examination of the tumors made it possible to distinguish two types, designated hereafter as Types I and II. Type I cells (Fig. 1) appeared to be actively and vigorously growing. They resembled the tumor cells described by Tweedell (12). The nuclei stained intensely with Feulgen, and the Feulgen-positive material was distributed throughout the nuclei. Chromocenters were more numerous than in normal kidney cells. Because of the lack of contrast with the nucleoplasm which also stained with Feulgen, however, the chromocenters appeared less prominent. In these cells it was impossible to distinguish nucleoli. In all tumors of this type mitotic figures were frequently seen. In some cases, as many as 20 per cent of the cells were undergoing cell division.

The cytoplasm of cells of Type I tumors stained fairly uniformly with fast green or with eosin. There were some necrotic areas in these tumors, and large aggregates of dead cells were often seen in the lumens. These aggregates consisted of spherical or crescent-shaped dead nuclei and amorphous debris. The dead nuclei stained very brightly and solidly with Feulgen. Spherical or crescent-shaped Feulgen-positive bodies were seen in the cytoplasm of many of the non-necrotic tumor cells. These bodies closely resembled the necrotic nuclei, which were free in the lumens. They were never attached or connected to the nuclei of the cell in which they were found. It seems likely that these cytoplasmic Feulgen-positive bodies were pyknotic nuclei which had been engulfed by healthy cells.

Of the 61 tumors examined 54 fitted the description of Type I tumor cells. All of these were obtained from frogs which had been maintained at 20°-25° C. with feeding.

Type II tumor cells (Fig. 2) resembled those just described with respect to cellular and nuclear enlargement, the columnar shape of the cells, and the pseudostratified appearance of the epithelium. These tumors, however, did not appear to be vigorous and actively growing as did Type I tumors, but resembled those described by Fawcett (2). The cells were generally necrotic and the nuclei stained lightly with Feulgen. The chromatin material was very sparse, and the nucleoplasm did not stain with Feulgen. The chromocenters were relatively small and were often concentrated along the nuclear membrane. Most of the nuclei in which the chromatin was not marginalized in this manner exhibited a very prominent Feulgen-positive nucleolar organizer in the center of the nucleus. This ring-shaped structure was similar to that seen in the nuclei of normal kidney cells but was larger and more prominent. In tumors of this type no mitotic figures were seen.

Some of the cells in which the chromatin material was found close to the nuclear membrane exhibited an irregularly shaped aggregate. This pleomorphic mass stained densely with fast green and was usually located in the center of the nucleus. The proportion of the cells in a given tumor which contained such fast green-staining aggregates varied between 1 and 50 per cent, but some could be found in every Type II tumor. The aggregates were not localized in any particular part of the tumors, but randomly throughout, and their frequency was not correlated with the size of the tumor. These aggregates were never seen in tumors of Type I, in normal kidney cells, or in normal tissue adjacent to Type II tumors in cases where it was possible to study adjacent tissue. A total of seven tumors examined fitted the description of Type II tumors. All seven had been taken from frogs which had been in a state resembling hibernation—i.e., they had been kept at 5° C. for more than 2 months without being fed.

There was no correlation of size of tumor or its degree of advancement or invasion into the adjacent tissue with the type of cytology. Since the age of the frogs was unknown, a correlation with age may have gone unobserved.

To summarize the main differences between the two tumor types, a predominance of necrotic cells and the presence of acidophilic intranuclear inclusions were apparent in every tumor (Type II) obtained from frogs kept at 5° C. In contrast, cells appearing to be “healthy,” with vigorous mitotic activity, and engulfed pyknotic nuclei in the cytoplasm of some cells (Type I) were seen in tumors from frogs kept at 20°-25° C. Type I cells were not observed in tumors from frogs kept at 5° C., and Type II cells were not seen in tumors from frogs kept at 20°-25° C.

**DISCUSSION**

*Transmission experiments.*—The experimental results show that there was no statistically significant difference between any of the groups of frogs in the proportion in which tumors developed after 4 months under the conditions described. It can be concluded that under these conditions whether or not a renal tumor developed in a frog during a period of maintenance in the laboratory of this duration was determined before the period of laboratory maintenance began, and that this
was not influenced to a significant degree by the various inoculations or by cross-infection among frogs in the laboratory.

Rafferty's results (11), as previously noted, indicated that there was an increasing proportion of frogs with tumors after an increasing period of time in the laboratory. In the light of the results of the present investigation, this increase is interpreted as resulting from the long latent period of the tumors, at least for the first several months of laboratory maintenance. The latent period elapsed for an increasing number of incipient tumors with increasing time in the laboratory, resulting in a rising tumor incidence.

The occurrence of tumors among frogs which had not received inoculations in this study was 20.6 per cent after 4 months. That among the uninoculated frogs maintained for 6 months by Lucké (5) was 6.5 per cent. The difference might be attributed to the fact that all the frogs in Lucké's experiment were housed at a lower temperature (7°-10° C. in winter, up to 18°-21° C. in summer). It is thus possible that at 25° C. such a high proportion of frogs develop tumors spontaneously that it is difficult or impossible to raise the tumor incidence by inoculation. Lower temperatures have been found to inhibit tumor formation as in the work of Rafferty (10), who found no tumors among 21 frogs maintained at 13.5° C. for periods up to 31 weeks.

There is another possible explanation for the difference in tumor incidence in uninoculated frogs. It is possible that the postulated virus or agent is more widespread in nature today than it was at the time Lucké's transmission experiments were performed. Tumor-free frogs of today may be resistant and therefore difficult to infect in nature or by experimental inoculation.

Cytological studies.—Acidophilic intranuclear aggregates have been seen in some cells of every tumor removed from frogs maintained at 5° C. Comparison with Fawcett's (9) photomicrographs indicates that they are the same as the nuclear inclusions he observed in four of the twelve tumors he examined. The temperature at which his frogs were maintained is not known. On the basis of electron microscopy he stated that virus-like particles were found only in tumors containing some cells with this nuclear alteration, and only in those cells in which the nuclear alteration appeared. Thus, I suggest that the virus is detectable as a morphological entity only in tumors from frogs in a state similar to hibernation. In the present study the nuclear inclusions were not seen in any of the tumors from actively metabolizing frogs (Type I tumors). If this observation is correlated with Fawcett's results (9), the virus particles may be presumed to be either absent or present in a different form in these tumors. In the light of this, it is of interest to recall Lucké's suggestion (5) that the nuclear inclusions were more frequent in the winter and spring. That would be when the frogs are hibernating or emerging from hibernation.

Type II tumors, which contained cells with the acidophilic nuclear aggregates and presumably the virus particles, appeared to consist of degenerating tumor cells. The nuclei stained very faintly, the cells appeared necrotic, and mitotic activity was rarely seen. The cells may have been degenerating as a result of exposure to low temperature and a long period of starvation; or, perhaps they were damaged by the presence of the virus. A possible effect of the metabolic conditions of hibernation may be to upset an equilibrium which otherwise exists between virus and tumor cells, allowing the virus to proliferate at the expense of the cells. On the other hand, the presence of the virus and the degeneration of the cells may be causally unrelated.

In either case, the problem of the relationship between the virus particles and the etiology of the tumor has yet to be resolved. This virus may be the etiological agent of the tumor, usually present in the tissue in a latent or undetectable form and activated by hibernation or conditions simulating hibernation. Moreover, it is possible that the virus is normally transmitted in nature during hibernation, the congregation of frogs at the bottoms of ponds at that time raising the population density of the species (9) and facilitating transmission. This factor could certainly contribute to the survival of the virus in nature. On the other hand, the virus seen in Fawcett's photomicrographs may be merely a "passenger virus," normally present in the tissues of frogs, having a special affinity for the cells of the tumor. The tumor cells may be able to resist infection by the virus except under these metabolically altered conditions.

The spherical Feulgen-positive body often seen in the cytoplasm of Type I tumor cells was identical in shape, size, and location with the cytoplasmic inclusions described by Tweedell (12). The tumors he examined were taken from frogs kept at 25° C., similar to those in this investigation. There was no indication in the tumors described here that this cytoplasmic Feulgen-positive material was in any way related to viral activity. The inclusions were morphologically similar to the pyknotic nuclei sloughed into the lumens of the tumor with cellular debris. The inclusions were probably necrotic nuclei which had been engulfed by intact tumor cells adjacent to dead ones, or by cells lining
the spaces containing necrotic material. That this was the case was further suggested by the fact that these cytoplasmic Feulgen-positive bodies were seen only in tumor cells from actively metabolizing frogs, such cells being more likely to be capable of phagocytosis than the degenerating cells of the other type. Cellular engulfment has been previously reported in the cells of normal, neoplastic, and ulcerated colon mucosa, normal uterine endometrium (7), and rectal polyps (3). The cytoplasmic inclusions in the Type I cells did not in any manner resemble the cytoplasmic alterations described by Fawcett (2).

The data presented here indicate that there is a need for re-evaluating the evidence that a virus is the etiological agent of the renal carcinoma of Rana pipiens. This study demonstrated that additional adequately controlled transmission attempts and further examination of the cytology are needed. Some of the findings cast doubt on the previous indications considered to be evidence in favor of the viral etiology of this neoplasm. They are the incidence of tumors in uninoculated frogs to a degree comparable with that in the inoculated ones of this and of previous studies (1, 5, 6) and the presence of the nuclear “inclusion body” in the tumor cells only under certain special conditions.

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Studies on the Transmissibility and Cytology of the Renal Carcinoma of *Rana pipiens*

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